Chapter 9
Precise Gene Editing of Cereals Using CRISPR/Cas Technology

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Abstract Targeted mutagenesis using CRISPR/Cas technology has become routine in elucidating biological processes or their application in breeding and agriculture. This means that the change to be achieved can be accurately predicted. However, knockout of a gene function is not always desirable, as reducing activity or affecting a protein domain can influence its properties and, thus, the phenotype. This chapter will therefore focus on precise genome modification in temperate cereals. The methods used, including some representative examples, are summarised here.

Time is an essential factor to consider in developing new agricultural varieties. Since domestication, plant breeders have steadily expanded their toolbox, but establishing a new cereal variety takes an average of 8–10 years [1]. Developments in genome sequencing (barley [2], wheat [3], rye [4]), oat [5], and molecular biology methods for genome-assisted breeding (marker-assisted breeding [6]) have provided tools and techniques for the breeding process that positively influence the process and workload.

In this chapter, temperate cereals of the botanical tribe Triticeae (barley, wheat, rye, and oats) grown in Europe will be considered. Due to limitations in the availability of genomic sequences (rye and oat only in the last two years) and the lack of efficient transformation protocols, there are currently only reports from barley and wheat. These are also among the more essential cereals in Europe in terms of cultivated area (FAO Stat). While diploid barley is mainly used for animal feed, beer, and whisky, tetra- and hexaploid wheat are essential for pasta and bakery products. Oat and rye are the main components of breakfast cereals.

Targeted mutagenesis induced by endonucleases such as TALEN [7] and CRISPR/Cas [8] has enabled an incredible number of applications in a wide range of species since their first biotechnological application in 2012 [9]. Thus, the results have helped many new insights into basic research and show promise for

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applications. Targeting an organism's genomic sequence has never been so easy. CRISPR/Cas technology is a two-component system in which a target-specific guide RNA guides a double-strand-inducing Cas enzyme to the desired location in the target genome [8]. The cell's repair mechanisms then repair the induced double-strand breaks. A more detailed description of the technology and repair mechanisms is described in Chap. 1.

Applications of precise genome editing in plants have been summarised several times (for review, see [10–12]). These reach into all areas of modern plant research. Starting with pure basic research, i.e., the identification and characterization of genes and their phenotypic expressions, the methods are also used to master the current challenges of agriculture. Here, agronomic parameters play a similarly important role in improving product characteristics. Plants with enhanced resistance to fungal or viral pathogens are being developed and tested. Plants should be better adapted to changing climatic conditions and thus have better water and nutrient utilization. Plants should become heat or drought-tolerant but also be able to grow with an increased salt concentration in the soil. There are examples of all these experiments in barley and/or wheat (Fig. 9.1). The only publication on applying CRISPR/Cas technology in triticale, a cross between wheat and rye, shows its functionality exclusively in protoplasts [13].

Even if the targeted induction of a double-strand break can be carried out precisely, the result is random. According to previous reports, the most common

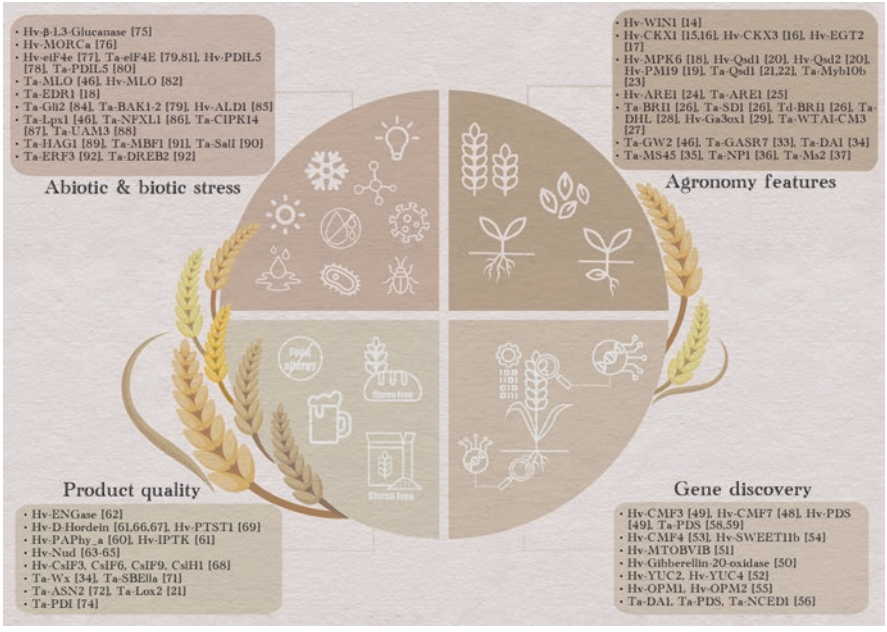

Fig. 9.1 Summary of barley and wheat genes with functions in agronomic features [14–47], gene discovery [48–59], product quality [21, 34, 60–74], and abiotic and biotic stress [46, 75–92]

outcome for CRISPR/Cas9 is a deletion of a few (≤10) nucleotides or the insertion of one nucleotide (InDel) [93]. However, it is impossible to predict precisely whether loss or insertion will occur, and it is also somewhat random which base is inserted. Although there are reports (for review, see [94]) that a certain percentage can be predicted using microhomology-dependent repair mechanisms, the outcome remains undetermined in most applications.

To achieve a prediction of the mutation result and thus precise genome editing, one can consider several possibilities. When using two gRNAs, one gets an exact deletion between the two induced double-strand breaks in a part of the mutated cells. In this case, the choice of gRNA binding sites can, for example, influence the function of a protein domain [49]. By not inducing a knockout, a reduced or altered functionality remains, similar to the RNAi effect but is genetically fixed in contrast.

Another possibility is to transfer a repair template with the desired sequence simultaneously with the double-strand-inducing reagent. However, the challenge here is to bring a sufficient number of repair templates to this site at the time of the double-strand break repair. One possibility is the biolistic transfer of the repaired DNA [95]. However, this method has all the previously described disadvantages that have led to the preferential use of *Agrobacteria*-mediated transformation [96].

Initial results at the cellular level in barley showed targeted allelic exchange of the fluorescence protein GFP. GFP and YFP differ in only one amino acid; thus, exchanging two nucleotides causes a change in the emission spectrum [97]. It was shown that 3% of the mutant epidermal cells had integrated the non-functional YFP fragment in the genome, thus exhibiting a shift in the lambda scan. These results were even surpassed when pre-assembled RNP complexes were biolistically transferred with Cas9 instead of plasmid DNA [95]. Here, it was shown that up to 8% of GFP-mutated epidermal cells exhibited such an allelic exchange. A typical application for allelic exchange is the creation of herbicide resistance [98]. Since this allows the selection of the correctly modified cells, such a method is easier to apply. However, the efficiency is expected to be lower if the modification has no selection advantage during creation.

An improvement here is the prime editing method [99]. In contrast to the Cas9 technology, the Moloney Murine Leukemia Virus reverse transcriptase (MLV-RT) domain was added to the Cas endonuclease. At the same time, the gRNA was extended by the part of the repair template. However, there have been few reports of plant applications so far, suggesting that the technology still needs improvement.

To precisely incorporate large DNA fragments in plants, a PrimeRoot-named method was recently described [100]. Third-generation PrimeRoot editors use optimized prime editing guide RNA designs, an improved plant prime editor, and superior recombinases to enable precise large DNA insertions of up to 11.1 kilobases into plant genomes. The authors describe using PrimeRoot to introduce gene regulatory elements into the rice. Applications in temperate cereals have not yet been described.

Base editing (BE) is another technology for the precise modification of genomes (DNA) or transcriptomes (RNA) of living cells at single-base resolution (for review, see [101]). BEs comprise a catalytically impaired cas nuclease fused with a

nucleotide deaminase and sometimes DNA repair proteins. BEs can introduce single nucleotide variants at desired sites into the DNA (nuclear or organellar) or RNA of both dividing and non-dividing cells. There are two types of BEs – DNA BEs, which directly induce targeted point mutations in DNA, and RNA BEs, which convert one ribonucleotide to another in RNA. The currently available DNA BEs can be further divided into cytosine BEs (CBEs), adenine BEs (ABEs), C-to-G BEs (CGBEs), dual-base editors, and organellar BEs. These categories are discussed below [101]. After protoplast testing, C to T substitutions was successfully detected in two heterozygous wheat plants [102]. To increase the efficiency, further improvements such as the NLS, crRNA, LbCas12a nuclease, adenine deaminase, and linker were undertaken, achieving up to 55% efficiency in stable mutants (*TaLOX* and *TaMLO*) [103]. In other plants, further improvements, such as placing a N-terminal reverse transcriptase–Cas9 nickase fusion performed better in rice than the commonly applied C-terminal fusion [104]. In addition, introducing multiple-nucleotide substitutions in the reverse transcriptase template stimulated prime editing with enhanced efficiency. Additionally, it was shown that using two pegRNAs that encode the same edits but target complementary DNA strands highly promotes the desired outcome [105].

However, all the previously mentioned methods are still subject to particular challenges. These concern all parts of the process, such as selecting, using, and transferring appropriate gRNAs and Cas and proteins, general tissue culture, genotype dependence, detection of induced mutations, and identification of transgene-free, etc. homozygous progeny [106]. Further sequencing of genomes and enzyme evolution will undoubtedly lead to other plant improvements. It is crucial that the plants produced in this way also find use in European agriculture and that outdated regulations do not prevent their use.

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